FINAL REPORT

Verifying Food Web Bioaccumulation Models by Tracking Fish Exposure and Contaminant Uptake

SERDP Project ER-1749

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Karl Gustavson
U.S. Army Engineer Research and Development Center

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LIST OF ACRONYMS

ACUP: Animal Care and Use Protocol
DoD: Department of Defense
EPA: Environmental Protection Agency
ERDC: Engineer Research and Development Center
IP: Intraperitoneal
IV: Intravenous
NOAA: National Oceanic and Atmospheric Administration
ORD: Office of Research and Development
OSU: Oregon State University
PCB: Polychlorinated Biphenyl
USACE: United States Army Corps of Engineers
SERDP: Strategic Environmental Research and Development Program

ABSTRACT

Objective

The *overall objective* of this project is to develop and use innovative technologies that eliminate fundamental uncertainties in food web modeling by unambiguously documenting contaminant exposure, chemical uptake, and growth over time in individual fish, and to use that information to verify and improve food web bioaccumulation modeling approaches.

The *limited scope objective* is to develop a fish tracking/recovery technology that monitors fish location over time and permits recovery of the fish at specified time points. The *go/no go criterion* is whether 50% or greater implanted fish can be successfully incapacitated and recovered from the water at the specified time point.

Technical Approach

The technology development was based on the testing, analysis, and improvement of prototype devices. The technical approach was 1) Review the scientific and patent literature to evaluate whether tracking technologies or other approaches in whole or part have been prepared that are capable of achieving device requirements; 2) Develop an initial prototype to initiate the cycle of iterative testing and refinement; 3) Test devices to assess whether they achieve the device requirements of tracking, euthanization, and surface recovery of implanted fish. Analyze results to improve device function and reliability; and 4) Evaluate whether the final device achieves the go/no go criterion.

Results

A prototype tag was designed that contained the power, timing, activation, inflation, and euthanization modules. The prototype was built and it achieved a size and weight that could be carried by fish without adverse impact. Iterative testing, modification, and improvement proceeded through four prototype versions to improve component performance, reliability, and overall form factor. Iterations of testing and improvement primarily addressed inconsistent performance of the flotation mechanism and early leakage of tag components that caused early mortality. Tag volume and weight were also reduced.

The final design possessed sufficient power, tracking capabilities, timed activation, and coupled flotation/euthanization agent release mechanisms. However, the euthanization efficacy was not tested due to insufficient progress with the prototype development. The current version possesses consistently proven power, tracking, and activation. Analyses identified why tags leaked and the flaw was isolated and fixed. The flotation mechanism is not yet consistently reliable and the euthanization mechanism is reliant on the flotation mechanism. The cause of the inconsistent inflation has been isolated and solutions have been identified. Overall, substantial progress was made toward developing a reliable and functioning tag design capable of achieving design requirements. However, the go/no go criterion was not achieved. To achieve objectives and go/no go criterion, the flotation mechanism needs to be modified and the coupled flotation/euthanization mechanism need to be verified in further prototype development and testing.

Benefits

If fully developed, these technologies will improve understanding of PCB bioavailability, accumulation, and biomagnification through the food chain and improve models used to predict the impact of sediment contaminants on higher trophic level organisms. These approaches will lead to more technically sound site risk assessments and, ultimately, expedite sediment cleanup and completion of liabilities by DoD.

OBJECTIVES

Limited Scope Proposal Objective

Develop a fish tracking/recovery technology that monitors fish location over time and permits recovery of the fish at a specified time point.

Overall Objective

Use developed device to improve food web bioaccumulation modeling at DoD contaminated sediment sites to facilitate more efficient and effective remedial decisions. The proposed work would establish clearer linkages between sediment and fish contamination by using innovative approaches and technologies to 1) eliminate fundamental uncertainties in food web modeling by documenting fish contaminant exposure (fish location, water/sediment contaminant concentration, and fish diet) and 2) use that information to verify and improve existing modeling approaches.

Criterion For Success

The proposal stated that "Development will be considered successful if implanted fish (50% or greater) are successfully incapacitated and recovered from the water body at the specified time point." For a proof-of-concept applicable to a field application, it was envisioned that a 30-day deployment period would be a reasonable duration for this proof of concept testing.

BACKGROUND

Foodweb bioaccumulation models are relied upon heavily in decision-making at DoD and other contaminated sediment sites. For PCB-contaminated sites, sediment remediation is conducted to decrease human health risk from consumption of PCBs in fish, not direct exposure to PCBs in sediments. So, the foodweb bioaccumulation models that establish the relationship between contaminants in fish and sediments are extremely important. The models are often used to estimate future fish tissue contaminant concentrations under various sediment remediation scenarios and their output can be the basis or justification for selecting particular remedial technologies. While foodweb bioaccumulation models' internal architecture is relatively complex and can consist of hundreds of input parameters (Thomann et al. 1992; Arnot and Gobas 2004; QEA 2005), relatively few empirical environmental data are input, consisting primarily of sediment contaminant and organic carbon concentration, water contaminant concentration, contaminant Kow, temperature, and sometimes diet composition (not considering fish tissue concentrations used in calibration and validation).

These models are often applied over wide areas (square miles of open water and/or miles of river) and a single sediment contaminant concentration is often assumed for the entire area, working under the assumption that fish will be exposed equally among all locations (von Stackelberg et al. 2002). It's understood that this is a poor assumption: fish movements are not random, surface sediment contaminant concentrations can be extremely heterogeneous, and fish

tissue concentrations reflect the concentration of the area of exposure (Bayne et al. 2002). However, there is often no basis for assigning spatially-explicit exposure patterns. At present, our fish tissue bioaccumulation models are calibrated and validated using fish with unknown provenance and exposure history. As a result, models used to relate sediment and fish contaminant concentrations are calibrated using fish that may or may not have accumulated contaminants from the area of interest. Indeed, not knowing a fish's exposure pattern is one of the greatest uncertainties in fish bioaccumulation modeling (Linkov et al. 2002). That uncertainty is a major obstacle to making informed decisions about remediating sediments to decrease fish tissue contaminant burdens (Chien et al. 2002; Moore et al. 2005). Fish tracking studies have assisted in establishing exposure areas of fish at Superfund sites (Myers et al. 2003; Myers et al. 2008), but they have not been directly interfaced with bioaccumulation studies.

Other obstacles in developing fish bioaccumulation models for making informed remedial decisions are establishing the growth rate of the organisms and bioavailability of the chemicals in the sediments. Often at sites, the weight/size of the species analyzed is available, but the weight of the species as a function of age is unknown. Weight-age class data are needed to establish the growth dilution rate constant (kg in the foodweb models). In the absence of data, that constant is estimated by allometric relationships based upon the weight of the organism. Growth is important because the concentration of the chemical in the organism is diluted by the new tissue. With the proposed approach, growth of the organism can be measured along with the increase in chemical residues in the organism. The comparison of the actual and expected chemical uptake by the organism allows the determination of bioavailability if the chemical across the foraging range of the organism is unusual. If unusual, the site conceptual model needs to address this issue prior to decisions on remedial actions.

The residue in an organism is defined by the comparative uptake and loss rates of the chemical by the fish, and in equation form (Arnot and Gobas 2004)

$$\frac{dC_{fish}}{dt} = k_1 \times C_w + k_d \times C_{food} - (k_2 + k_g + k_e + k_m) \times C_{fish}$$

where Cfish concentration of the chemical in the fish
t time in days
k1 gill uptake rate constant (1/day)
Cw concentration of bioavailable (freely dissolved) chemical in the water
kd dietary uptake rate constant (1/day)
Cfood concentration of the chemical in the food (prey items including sediment)
k2 gill elimination rate constant (1/day)
kg growth dilution rate constant (1/day)
ke fecal egestion rate constant (1/day)
km biotransformation rate of the chemical in the fish (1/day)

In bioaccumulation models, there is a set of equations like the one above; one for each species with its own set of parameters. The innovative approach reduces the uncertainties associated with exposure concentrations (Cw and Cfood), organism growth rate (kg), and chemical bioavailability. Chemical bioavailability influences the calculation of kd and ke due the digestion processes in GI track. Digestion sub-models track the changes in lipid, water, and

non-lipid in the food consumed by the organisms, and ultimately, derive the kd and ke rate constants based upon these changes.

Tracking devices can also measure temperature, another important input parameter to food web models, which in many cases is modeled using an average yearly temperature or seasonal temperature profiles because of the lack of data. Temperature is used in estimating respiration and feeding rates of the organisms, and ultimately, influencing the k1, k2, and kd rate constants in the Arnot-Gobas model (Arnot and Gobas 2004). Additionally, temperature is used in selecting the allometric equations for estimating organism growth dilution rates (kg). The tracking devices will directly map the temperature profile of the organisms at the site of interest according to their specific exposure history. Many organisms have preferred temperature conditions and the tracking devices will reveal these patterns when present. Using the actual temperature profiles will lower uncertainties in predictions with the food web models because average yearly or seasonal temperature profiles are not used.

The overall hypothesis is that innovative approaches and technologies proposed herein can be developed that eliminate fundamental uncertainties in food web modeling by documenting contaminant exposure, chemical uptake, and growth over time in individual fish. Bioaccumulation modeling predictions should be verifiable (Luoma and Fisher 1995)—the studies and technologies proposes herein would collect highly-specific fish contaminant and exposure data to permit that verification which could then be used to improve model structures and application at sites.

Benefits: The proposed approach would eliminate fundamental uncertainties in the modeling of fish bioaccumulation of PCBs and provide a detailed understanding of the environmental exposures driving contaminant uptake. The approach would clearly document contaminant exposure (fish location, water/sediment contaminant concentration, and fish diet), and provide detailed contaminant uptake kinetics. That information, along with data on fish growth and temperature, would be used to verify and improve bioaccumulation modeling, improving site specific understanding and model performance in general. This level of information provides far greater value and insight than current techniques where model application assumes fish are exposed equally across broad exposure areas, fish diet compositions are estimated, and fish used in model calibration and validation have unclear exposure histories.

If developed, the proposed devices would likely find wide use in research applications beyond those proposed, such as evaluating the effects of short-term events (remedial actions, storms, spills) on contaminant uptake, studies of interspecies differences in biomagnification through the food chain, and evaluations of movement patterns and contaminant dynamics in resident fish.

Ultimately, the proposed work would expedite sediment cleanup and completion of liabilities by the DoD and other federal agencies by clearly establishing the relationship between sediment and fish tissue contaminant concentrations. Better establishing that linkage will be a tremendous asset to decision makers seeking to determine the environmental compartment, location, and magnitude of contamination that requires remediation to achieve risk-based fish tissue contaminant concentrations.

MATERIALS AND METHODS

Device Requirements

To achieve the limited scope and, ultimately, the overall objective, a fish tracking and recovery device design needs to fulfill three primary requirements.

1. Track fish movement over deployment period
2. Euthanize fish at a specified timepoint
3. Transport fish to surface for recovery

Device Design Criteria

In fulfilling these requirements, there are several criteria that must be met for a device design to have long-term success:

- Euthanization will be rapid, effective, and result in minimal pain perception by the tagged fish.
- Design will minimize safety and liability concerns associated with handling or ingestion of a tagged fish or parts thereof by humans or other animals.
- Device or implantation procedure should not influence the behavior of tagged animal.
- Device design and material will be biocompatible to minimize stress and avoid inflammatory responses.

Approach

The original proposal lays out four tasks for the project (Table 1).

Table 1. Project Tasks.

Task	Explanation
1. Review existing tracking devices and potential to incorporate immobilization technologies.	Review literature to evaluate whether tracking technologies or other approaches in whole or part are capable of achieving device requirements.
2. Conduct prototype development.	Initial prototype will be developed to initiate the cycle of iterative testing and refinement envisioned in Task 3.
3. Prototype testing and refinement.	Devices will be tested to assess whether they achieve device requirements. Modifications will be made based on testing to improve device function. A final tracking/recovery device will be prepared for Task 4.
4. Final mesocosm-scale testing of devices to prove recovery of fish at anticipated timepoint.	Final testing is intended to be proof-of-concept for the developed device that it is able to achieve the requirements of tracking, euthanization, and surface recovery of implanted fish.

A number of individuals and groups participated in the development, manufacturing and testing of the devices. The major participants and their roles and responsibilities are described in Table 2.

Table 2. Project Team.

Participant	Role
Army Engineer Research and Development Center. Karl Gustavson	Principal Investigator. Advise on device design, use, function, needs, and testing from a contaminated sediment management perspective.
Lotek Wireless. Mitch Sisak, Neil Bower, and Marek Pach	Engineer, manufacture, assemble devices. Ex-vivo testing of prototype devices.
Oregon State University (OSU), Oregon Cooperative Fish and Wildlife Research Unit. Carl Schreck and David Noakes	Implant and test developed devices in fish at the hatchery facility under approved animal care and use (ACUP) protocols.
U.S. Environmental Protection Agency. Lawrence Burkhard	Expertise in bioaccumulation modeling for evaluating uptake of contaminants in fish from sediments.
National Oceanic and Atmospheric Administration, Northwest Fisheries Science Center. Lynn McComas	Expertise on field implementation of fish tracking studies, tag implantation, and fish behavior.

RESULTS AND DISCUSSION

In this section, details and experimental results are provided for each task outlined in Table 1. A summary section is provided under each task to succinctly convey results and discuss their implications and relevance in addressing the project's objectives.

Task 1. Review Existing Tracking Devices and Potential to Incorporate Immobilization Technologies.

Device requirements as specified above were reviewed to evaluate whether they could be satisfied by current technologies. Reviews included literature, patent, and internet surveys and discussions among device manufacturers and fish tracking experts.

Requirement 1: Track fish movement over deployment period

Acoustic transmitters have been widely used to track fish movement for several decades (Sibert and Nielsen 2001; Lembo et. al., 2002). Lotek Wireless, the fish tracking device manufacturer partnered with on this project, designs and manufactures acoustic transmitters and receivers for tracking fish in a variety of environments and applications. Thus, *de-novo* technology development is not needed to satisfy Requirement 1.

Requirement 2: Euthanize fish at a specified timepoint

Fish tracking devices have not been adapted to immobilize fish and permit fish recovery. Extensive protocols and procedures exist in the animal care and welfare literature for the humane handling, stunning and killing of fish, but they apply almost exclusively to commercial or laboratory conditions (CCAC, 1984; Stoskopf, 1993; Close et. al., 1996). Following a literature and online review, no information could be found covering the in-situ remote dispatch of fish, so accepted procedures (CCAC, 1983; Lines et al. 2003; EFSA, 2004) will be incorporated into the design of the tracking and immobilizing tag being developed.

The remote euthanization of a tagged fish will require a timer/activator and a euthanizing mechanism. Possible mechanisms of euthanasia include:

- physical: producing severe trauma
- electrical: producing disruption of normal muscular or CNS activity and
- chemical: employing poisons or homeostasis disrupters

The suitability of these mechanisms for the tag under development was evaluated from a number of perspectives. These included the stability and reliability of its components for the duration of the deployment period. In the case of toxins, changes in toxicity over time were considered. The ability to deliver sufficient physical trauma or the appropriate electrical charge was also explored as well as the mechanism's ability to provide long term reliability. Other factors explored were related to animal health and welfare. For example, at the time of activation, the selected mechanism will be required to rapidly dispatch the animal in a manner which would limit pain perception. Material selection and design will be made with an eye on biocompatibility to minimize any stress associated with inflammatory response. Safety and liability issues were also considered in an effort to minimize issues associated with either the handling or ingestion of a tagged fish or parts there of by humans or other animals. These include but are not limited to trauma, electrocution, poisoning or burns. Finally, the complexity of the installation of the euthanizing device to ensure its reliable operation was considered. For example, if an electrical charge applied to the heart was evaluated as a potential euthanizing mechanism, the complexity associated with implanting the required electrodes, the time required to effect those connections and the likelihood of those connections being maintained over a 90 day deployment were considered.

Requirement 3: Transport Fish to surface for recovery

Fish recovery at the water surface is the most practical option for retrieval. Mechanisms were explored to achieve and maintain positive buoyancy at the specified time for a reasonable window of recovery. An extensive review of the biotelemetry literature and a patent search produced only one device designed specifically to permit the recovery of a tagged fish during turbine passage mortality studies (Normandeau-Associates and Skalski 2003). Here, fish recovery is facilitated by an inflatable float device tethered to the fish. The flotation mechanism activates shortly after deployment which does not lend itself to longer term studies. The external, tethered attachment would likely sustain damage over the intended mission time. Further, the possibility of entanglement posed by a tethered device as well as the negative impact

on normal behavior of tagged individuals through modified hydrodynamics makes this device unsuitable for our longer term applications.

An internally inflated bladder would avoid the above-noted constraints. A surgically implanted device would be in a stable and protected environment, improving reliability of the device over the required deployment period. If a device of suitable size and weight could be produced, its presence inside the peritoneal cavity would result in minimal impact on the behaviour of the tagged fish (Bridger and Booth, 2003; Lacroix *et. al.* 2004). The increase in buoyancy required to cause an inanimate fish to rise to the surface was found to be small, requiring only a minimal change in specific gravity (Taylor, 1922). Discounting any assistance from a gas bladder or other naturally occurring organ or mechanism, the average fish has a specific gravity of between 1.06 and 1.09 (Bond, 1979) very close to the specific gravity of fresh water and saltwater which are 1.00 and 1.026 respectively. Thus, a change in specific gravity could be achieved through the inflation of a relatively small internally mounted bladder.

The suitability of proposed inflation mechanisms were evaluated employing similar factors to the Device Design Criteria above. Several combinations of chemicals of low toxicity have been identified which could be employed to generate a volume of gas sufficient to inflate a bladder, rendering the fish positively buoyant. To prevent adverse effects to the tracking module, reagents should be contained separately within the body of the tag. Triggering of the activator would result in the mixing of the chemicals and the evolution of gas, inflating the bladder and causing the fish to rise to the surface. A bladder constructed of low permeability and high puncture resistance material would remain fully inflated within the peritoneal cavity for a period of time sufficient to recover tagged fish. An expected side benefit associated with the mechanical expansion of the bladder would likely be capillary breakage within the peritoneal cavity that would enhance the uptake of the euthanization agent.

Combining Tracking, Euthanization, and Flotation Modules

While reasonable and achievable mechanisms were identified for each individual requirement, when combined, the resulting tag must maintain a size and form factor that does not adversely influence the behavior of the tagged fish. Adams et al. (1998) indicated that an appropriate tag-weight to body-weight ratio for active tag implants in juvenile salmonids should not exceed 5% of the animal weight. Other research (Lacroix et al. 2004) recommended that transmitter weight be kept at less than 8% of body weight for juvenile Atlantic salmon. Therefore, the targeted maximum tag-to-body-weight ratio was 5-8%. Thus, the size of fish used in a field application would also be a function of tag size.

Task 1 Summary

It was clear from literature searches, online reviews, and discussions with device manufacturers and fish tracking experts that tags capable of fulfilling Device Requirements were not readily available. The following strategy was selected based upon a review of existing tracking devices, immobilization technologies, device requirements, and design criteria:

- The device will be surgically implanted to protect, simplify, and maximize euthanizing efficiency and reduce overall stress to the animal related to the attachment procedure or the physical presence of the device.

- Tracking, euthanasia, and recovery functions will be achieved by the following device components and sequence:
  1. Each tag will contain a coded acoustic transmitter for unique identification of each animal (tracking function) and a microcontroller programmed to activate a low-power pyrotechnic squib actuator at a predetermined time (activation).
  2. The actuator will initiate a chemical reaction to generate gas and inflate a miniaturized and rugged bladder.
  3. Upon inflation, the bladder serves the dual purpose of expelling the euthanization agent into the intraperitoneal cavity of the fish and floating the fish to the surface.
  4. Chemicals and construction materials will be selected to minimize animal care and welfare concerns and any inadvertent adverse effects to possible human or wildlife predators.

Task 2. Conduct Prototype Development.

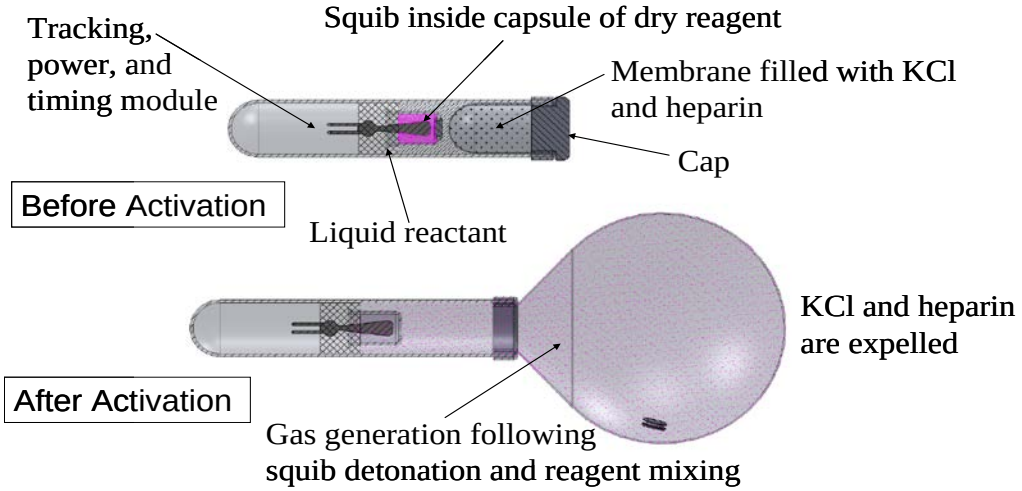
A proof of concept prototype device was developed that employs the strategy presented in Task 1. As described in Task 3, the refinement process altered the design of the inflation mechanism, but the general device components, presented below, remained consistent.

Device Components

The prototype device consists of 3 basic components: device housing, electronics module, and a payload.

The housing for the prototype device is a cylindrical tube, measuring approximately 100mm long and 16mm in diameter (later decreases in size were achieved). A drawing of the prototype appears in Figure 1. The materials selected protect the device modules until activation and payload deployment. Careful attention has been applied to ensure biocompatibility of materials used. The Version 1 prototype device weight was approximately 25 grams. For reference, this device would be approximately 5% of the weight of 1-pound fish, the lower end of the tolerances mentioned above. Reductions in weight and size of the device occurred as part of the device refinement process undertaken during Task 3.

Figure 1. Prototype Concept (Version 1 Tag)



The electronics module contains all the components responsible for the timed deployment of the payload, as well as the transmitter and transducer which comprise the acoustic transmitter portion of the device. The microcontroller responsible for triggering activation of the payload module contains an RTC (real time clock), that allows programmed deployment at a pre-determined time. A 3.0V battery powers the electronics module. Prior to activating the payload module, tag electronics operate in a state of minimal current draw, or sleep mode. During this period, scheduled acoustic transmissions take place that enable acoustic tracking. At the onset of an acoustic transmission sequence, the tag is brought out of sleep mode, an acoustic transmission is completed, and the tag is restored to its minimum current state. This procedure cycles until the activation time is reached and the payload module is activated. At this stage, acoustic transmissions cease and the payload deployment triggering sequence is initiated. Low current pulses from the onboard battery are directed to three high-capacity, parallel storage capacitors until sufficient charge is stored for squib activation. This design results in minimal strain on the onboard battery. Once the squib has activated, acoustic signal transmission resumes at its programmed time interval (a feature included to increase the likelihood of recapture of released individuals).

The payload consists of an inflation and euthanization module, both of which are triggered at activation. Payload deployment in the prototype tracking and immobilizing device is accomplished by triggering of a small, pyrotechnic 'squib', which is surrounded by dry reactant, sealed within a capsule. The capsule is then surrounded by a reagent sealed within the tube by an inverted flexible diaphragm. The euthanizing cocktail is contained within the inverted diaphragm. The open end of the device housing is sealed with a plug, which serves to protect the euthanizing cocktail from infiltration.

Device Details and Function

At the specified activation time, the capacitor bank fully charges and an FET (field effect transistor) switch is opened, allowing high current flow which serves to trigger the squib and initiate the payload module sequence.

Squib activation mechanically forces rapid mixing of the reagents resulting in gas evolution. The rapidly evolving gas causes the inverted diaphragm to be expelled from the device housing, ejecting the plug and euthanizing agent from the end of the device housing into the peritoneal cavity of the fish.

A number of chemical agents were considered and tested for the purpose of generating sufficient gas to accomplish the proposed task. The prototype used a two-part rigid foam resin (Pur Foam Rigid[1]) as the reactant and reagent. In this configuration, the two part resin mixes during the initial phase of the deployment sequence and progressively expands to fill the inflated volume of the latex. The diaphragm expands to achieve positive buoyancy.

The euthanizing agent is located in the tag housing in a pocket of the diaphragm isolated by a cap. The expanding diaphragm pushes the cap and agent into the body cavity of the fish

[1] Hardener: 4,4'-diisocyanate diphenylmethane ; Foam: Tris (2-chloro-1-methylethyl) phosphate; benzyldimethylamine; 2-phenylpropene, alpha-methylstryene.

upon activation. Potassium Chloride (KCl) was selected as the euthanizing agent because of its benign characteristics (at least via the ingestion and dermal exposure pathways) to non-target receptors. Tests were undertaken in Task 3 to evaluate the effectiveness of KCl delivered in this manner for euthanizing fish.

Task 2 Summary

Prototype development resulted in a design incorporating several functions/modules: power, timing, activation, inflation, and euthanization. The prototype design could achieve a tag of size and weight that could (based on literature review) be carried by fish of sufficient size without adverse impact. Prototype devices were prepared for testing and refinement as specified in Task 3.

Task 3. Prototype Testing and Refinement

Tag functions (power, activation, tracking, flotation, and euthanization) served as testing endpoints in Task 3. Quantities and type of euthanasia cocktail required to ensure rapid euthanasia were also evaluated. An iterative testing and modification program was envisioned to improve component performance and overall form factor.

The prototype tag and its successors were tested by the Oregon State University (OSU), Oregon Cooperative Fish and Wildlife Research Unit and by Lotek Wireless. In-vivo testing was conducted exclusively by OSU under an Animal Care and Use Protocol (ACUP) approved by OSU's Animal Care and Use Committee (See Appendix 1). Four iterations of the tag were tested before final testing (see Task 4. Final Testing, below). The tags and the testing results are described in this section. A table summarizing results from these versions is presented at the end of this section.

All in-vivo tests were conducted on adult, approximately 1-1.5 lb, rainbow trout (*Oncorhynchus mykiss*). These fish are generally tolerant of holding and handling procedures and were approved for use under Care and Use Procedures at the Oregon Hatchery Research Center. Selected fish were the approximate size appropriate for field study. Rainbow trout are not particularly robust fish, so they were considered good surrogates for evaluating whether developed tags would be tolerated by other test species.

Version 1 Testing

Version 1 testing focused primarily on the reliability and reproducibility of the power and activation modules and fish response to the tag over the deployment period.

Purpose. Version 1 testing evaluated the following endpoints:

- tag biocompatibility (no localized reaction to tag material),
- tag design compatibility (no adverse effect to organisms from carrying tag),
- reliability of activation,
- integrity of the fish insertion point following various periods of recovery (would ballooning tag rupture and eject from side of fish?), and
- flotation of fish following tag activation.

Setup. Three groups of two fish each were used. These groups were programmed to activate at 7, 10, or 13 days. During Version 1 testing, KCl was being investigated for its effectiveness as a euthanization agent (next section), so these tags were not loaded with the euthanization agent. Per the ACUP protocols, fish were euthanized in MS-222 just prior to expected activation time so that fish would not experience additional stress during activation. Therefore, euthanization was not one of the evaluated endpoints.

Results. All fish with implanted tags survived until the activation time. At the programmed time (7, 10, or 13 days post-implantation), all tags activated as designed. There was no disruption of sutures or expulsion of tags from the fish following activation. Fish did not float to the surface as expected. Upon removal of tags, it was verified that tags did activate, but balloon inflation was less than anticipated. Further, there was some evidence of discoloration of the flesh at the implantation site in the latter group fish, presumably resulting from leakage of reagent used in the foam mixture.

Conclusions. From these results, it can be concluded that weight and size of the Version 1 tags were well tolerated by the implanted fish. However, irritation at the site of implantation, due to exposure to the reagent in some tags, suggests that the reagent in combination with the housing would not prove biocompatible in the long term. The power and timing modules performed as expected with the squibs activating on time, indicating the successful design of the power and timing module. The inflation mechanism did not perform as expected and flotation did not occur following activation, indicating further refinement was needed.

Tag Modifications. Based on the Version 1 tests, two design modifications were decided upon. First, the generation of foam and resulting increase in buoyancy did not meet requirements, so acetic acid and sodium bicarbonate were selected to replace the two part foam mixture. Per device design criteria, these constituents are relatively benign and the mechanism of gas generation is robust and well understood. Second, to guard against premature leakage of components from the device a polyisoprene (synthetic latex) bladder was developed to surround the tag. Similar materials and applications have been used in fish without adverse reactions being noted.

Potassium Chloride Efficacy Testing

In conjuction with Version 1 testing, OSU completed a series of tests to establish the efficacy of potassium chloride (KCl) as a euthanization agent. KCl is a well known euthanization agent, causing rapid cardiac arrest when administered intravenously (IV) (AVMA 2007). However, intravenous or intracardiac injection is practical only under controlled laboratory situations, so it is not a reasonable route of administration in our application. The tags developed under this proposal were designed to expel KCl into the intraperitoneal cavity upon activation and inflation.

To assess KCl euthanization efficacy, it was administered to fish that had been anesthetized with MS-222 under controlled laboratory conditions per the ACUP. Cessation of opercular respiratory movements was used to establish the time until death and the lethal dose of potassium chloride. Rainbow trout were used as the study species, as it is widely used in field

and laboratory studies. Further details are provided in the Animal Care and Use Protocol (Appendix 1).

Results from testing fish injected with KCl via intraperitoneal (IP) and IV routes showed that KCl will need to enter the bloodstream to induce rapid euthanization. Fish dosed solely by IP injection of KCl were not killed within 5 minutes at doses carried by the prototype tags. Direct injection during tag activation is not feasible, so tag design will need to cause capillary breakage and allow KCl to enter the bloodstream. It was not tested whether KCl administered IP will have an acutely toxic effect over longer periods of time. That is quite possible, however, the mechanism of death would likely be a more slow and stressful death from osmotic stress relating to the high salt content.

Version 2 Testing

The version 2 tag incorporated the acoustic tracking module. In this design, the squib is embedded within the sodium bicarbonate, in a chamber adjacent to the acetic acid. The polyisoprene bladder surrounds the entire tube (Figure 2). Figure 3 depicts the tag post-activation. Note that KCl, the euthanizing agent, lies outside the bladder, isolated in the tube body by the cap.

Figure 2. Version 2 Tag Schematic

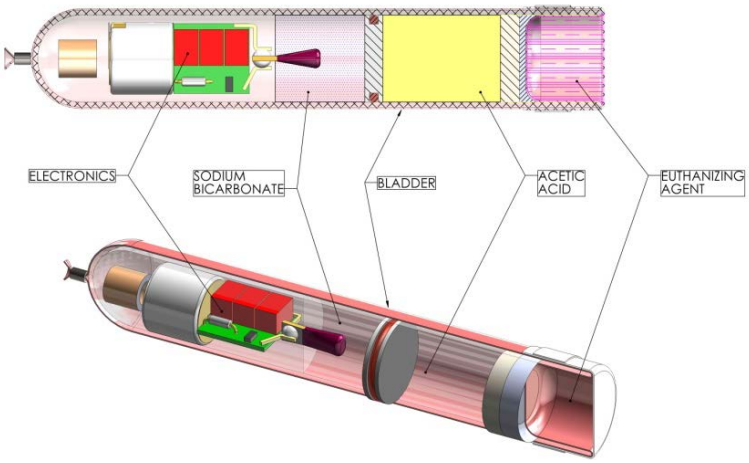
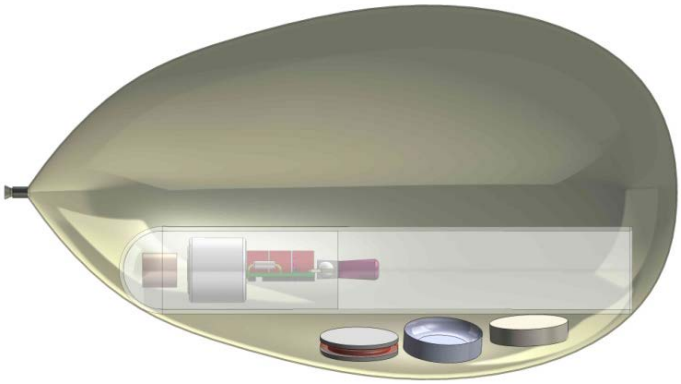


Figure 3. Version 2 Tag Post-Activation



Purpose. Version 2 testing evaluated the following endpoints:

- tag biocompatibility (no localized reaction to tag material),
- tag design compatibility (no adverse effect to organisms from carrying tag),
- reliability of activation,
- acoustic transmission of tracking module,
- flotation of fish following activation,
- euthanization of fish following activation.

Setup. Four fish were implanted with tags scheduled to activate at 10 days. A control fish underwent surgery but did not receive a tag. KCl was loaded in these tags so euthanization was to be evaluated.

Results. Tags actively transmitted acoustic signals, indicating successful incorporation of this module. However, within 24h, the three fish with implanted tags died. A failure analysis was conducted to determine the cause. The most likely fault scenario suggests premature leakage of acetic acid through the distal acetic acid barrier, damage to the bladder material, and eventual escape. The rapid fish morbidity combined with the reported smell of acetic acid implicates this event sequence. Detailed analysis of the prototpye tags by Lotek confirmed this scenario.

Conclusions. Clearly, the version 2 configuration is not suitable for use. The acetic acid in this design is highly susceptible to leakage. Upon death of the fish, the tests were terminated so additional endpoints (tag activation at specified time and flotation) were not examined.

Tag Modifications. In an effort to ensure the long-term isolation of the acetic acid from the other components of the tag, Version 3 tags were modified to house the acetic acid within a glass ampule. The ampule is surrounded by $NaHCO_3$, decreasing the likelihood of inadvertent exposure to the acid. Squib detonation is designed to rupture the ampule, liberate the acid, and permit mixing the $NaHCO_3$. Inflation of the bladder and delivery of the euthanizing agent occurs as described previously.

Version 3 Testing

The version 3 tag was the first design that compartmentalized the acetic acid in a glass ampule. It was believed that this design would prevent premature mortality.

Purpose. Version 3 testing was designed evaluate the following endpoints:

- tag biocompatibility (no localized reaction to tag material),
- tag design compatibility (no adverse effect to organisms from carrying tag),
- reliability of activation,
- flotation of fish following activation
- euthanization of fish following activation

Setup. Three fish were implanted with tags that were scheduled to activate at 10 days. One control fish was implanted with a “dummy” tag, which was of the size and shape of the actual tag, also contained in a polyisoprene bladder. Another control fish underwent surgery but did not receive a tag. KCl was loaded in these tags so euthanization was to be evaluated.

Results. One of the tagged fish died within 24 hours of surgical implant; the remaining three survived to the activation time, but the inflation mechanism did not fully respond. The "dummy" tag fish survived through the test and maintained normal behavior.

For the fish that died shortly after implantation, the tag exhibited partial ballooning. This issue appears to be a result of the ampule sealing process. When the acetic acid ampule is heated and sealed, a small pinhole diameter opening remains. For the Version 3 design, paraffin/dental wax was used to seal the opening. It is probable that exposure to heat during shipment and or storage reduced or weakened the paraffin seal in one of the tags allowing partial mixture of the acetic acid and the sodium bicarbonate. It is believed that the partial ballooning (and possible leakage of acetic acid out of the membrane) resulted in the early fish death.

The remaining three fish appeared healthy and behaved normally until time of activation. At that time, the tags activated (power module prompted squib detonation), but the tags did not inflate. The Version 3 tag was the first iteration to use a glass ampule, and a mesh 'sock' was used to cover the vial as a preventive measure against dispersal of glass shards and possible puncture of the inflated balloon. It was believed that the sock inhibited vial breakage upon squib detonation (in hindsight, the sock probably contributed to the lack of mixing, but was not the sole cause). Tags were disassembled following testing and it was determined that the ampules containing the acetic acid cracked but did not disintegrate. All three tags demonstrated some sign of chemical mixing, but that was not sufficient to initiate the inflation/euthanization sequence.

Conclusions. The Version 3 design was an improvement over Version 2. Inclusion of the glass ampule to contain acetic acid was partly successful at isolating the acetic acid, though one tag still leaked, a result of the ampule sealing process. The design also inhibited mixing of the acetic acid and sodium bicarbonate following activation. Version 3 testing proved that the membrane surrounding the tag was biocompatible and the size, weight, and form were "fish friendly".

Tag Modifications. Following version 3 testing, the tag was modified in an effort to increase acetic acid and $NaHCO_3$ mixing and to better sequester the acetic acid. In Version 4, the "sock" was no longer used. The paraffin sealant was also replaced with a cyanoacrylate based sealing material to fill in the micro pores found in heat sealed end of the vial. The ampule was also modified in two ways. First, to promote glass vial breakage, a deep dimple was formed at the point where the squib contacts the vial; second, the squib was cemented directly to the vial surface to facilitate fracture of the vial upon detonation. Figure 4 shows a schematic of the Version 4 tag and its components.

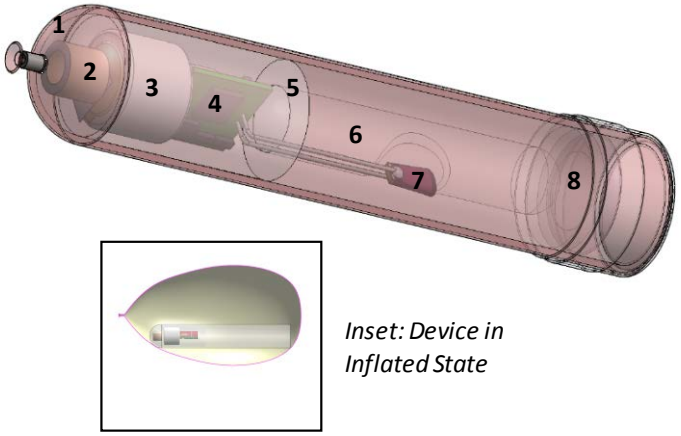
Version 4 Testing

Due to the inconsistent tag inflation and the early mortality event in version 3 testing, the version 4 tag testing was conducted ex-vivo to monitor tag performance and reliability.

Setup. Eight tags were developed for testing. Four were tested by Lotek and four by OSU. These tags included all modules (power, activation, tracking, inflation, and euthanization). The tags in each group were programmed to activate at 10 days post initiation.

Figure 4. Schematic of Version 4 Prototype

1. Latex Bladder around Tube
2. Piezo Ceramic Transducer to Enable Acoustic Transmission
3. Battery
4. Electronics Circuit Board
5. Sodium Bicarbonate
6. Glass Ampule Containing Acetic Acid
7. Squib Adjacent to "Dimple" in Ampule
8. Cap Containing KCl



Inset: Device in Inflated State

Results. The four tags tested by Lotek were fully successful in achieving all endpoints. These four tags actively tracked throughout the deployment period. All four tags activated at the specified time, floated to the surface, and discharged KCl. Figure 5 shows these tags before and after the activation sequence. The four tags tested by OSU actively tracked throughout the deployment period. All four tags activated at the specified time. Two of the four tags floated to the surface and discharged KCl. The two remaining tags did not inflate. Analysis of the tags without inflation indicates insufficient mixing of the acetic acid and sodium bicarbonate. Early leakage of acetic acid was not indicated in any of the tags tested by either group.

Figure 5. Version 4 Tags Prior to and After Inflation. Tags deployed as specified at 10 days.

Conclusions. The power, tracking, and activation modules of the tags performed fully and consistently in both groups of version 4 tags. The acetic acid leakage issue appears to be resolved (evaluated by monitoring pH of aquaria and by smell). However, 2 of the 8 tags failed to float following activation. This indicates the tag design is still not fully mixing the acetic acid and sodium bicarbonate. A post-experiment deconstruction of the tags showed the ampule was not being ruptured by the squib detonation during activation.

Tag Modifications. No modifications were made to the tags following these experiments since there was not sufficient time to re-engineer tag design. At this point in the program, OSU was unable to continue in-vivo testing. Due to time and resource constraints, the Version 4 design was brought forward into final testing.

Task 3 Summary

As planned, Task 3 was an iterative process of tag testing, modification, and improvement. During the course of Task 3 testing, there were four primary design modifications. First, the reactant/reagent mixture was changed from "pur-foam" to acetic acid and sodium bicarbonate. Second, a bladder was added surrounding the tag to facilitate flotation and prevent leakage of contents. Third, a glass ampule was added (and revised) to prevent acetic acid leakage seen in Version 2 and 3 tags. Finally, the tag volume was reduced approximately 23% and the tag weight by 16%.

Test endpoints show an incremental improvement over the course of tag development. However, setbacks in design modifications and in-vivo testing resulted in a Task 3 tag design that was not proven to meet the device requirements. Table 3 lays out the endpoints assessed in each versions' testing and whether they were achieved.

Table 3. Summary of Task 3 Tag Testing Outcomes

Version	Power	Tracking	Activation	Biocompatibility	Compatible Form	Flotation	Euthanization
1	Yes	NT	Yes	Yes (some internal adverse reaction noted)	Yes	No	NT
2	Yes	Yes	NT	No	NT	NT	NT
3	Yes	NT	Yes	Yes 2/3; No 1/3	Yes	No	No
4	Yes	Yes	Yes	NT	NT	6/8	NT (KCl release [ex-vivo] in 6/8)

NT: not tested

Task 4. Final Device Testing

Purpose

When the proposal was generated, the final testing was envisioned in live fish following a 30-day implantation period. The go/no go criterion was 50% of fish recovered from the water surface at the specified time point. In-vivo testing was discontinued due to time and resource constraints and animal care and use concerns stemming from previous "early adverse events" due to leakage of acetic acid from the tags. Thus, the original experimental design could not be pursued. In lieu of that testing, ex-vivo testing was designed to simulate in-vivo endpoints and provide proof of concept.

The final test of the prototype tag design involved a performance evaluation of a 14 day simulated deployment of 10 tags. A 30-day deployment period could not be used due to time constraints. The evaluation criteria were:

- Acoustic transmission
- Programmed device activation at specified time
- Ability to float implanted fish to surface; or, inflation and flotation of tag
- Ability to dispense the euthanization agent upon activation

Early leakage of acetic acid was also monitored to assess whether tag design modifications had addressed those issues.

Setup

As with all versions of the device, the countdown timer is initiated through the removal of an external magnet prior to tag deployment. To provide a detailed record of this test, all activities conducted during its execution were documented using time-stamped digital video.

While one of the evaluation criteria was whether tagged fish were rendered positively buoyant, it was also recognized that observing tags throughout the activation process would be advantageous. This would allow visual confirmation that the cap discharges and disperses KCl (euthanization itself could not be tested, so discharge of KCl was monitored). Therefore, it was decided that 5 tags would be implanted into dead fish placed in an aquarium to monitor flotation of a fish, while the remaining 5 tags would not be implanted into fish and simply placed underwater in an aquarium to monitor tag flotation and KCl release.

The prototypes are designed to be surgically implanted in a fish, so ex-vivo testing sought to recreate that environment. To avoid the obvious issues related to using dead fish for a 14-day experiment, initiated tags were stored in a 'simulated peritoneum' that would approximate real deployment conditions. This environment was created using a wetted (50ml) sponge sealed within a plastic envelope. The tags were divided into 2 groups of 5 tags: (A-1 through A-5) were non-implanted; (B-6 through B-10) were implanted. Tags in each group were initiated in sequence, with a slight delay between each.

Non-Implanted Group. One hour prior to the programmed activation time the non-implanted tags were removed from the "simulated peritoneum" and the tags were placed in a water filled aquarium. A video camera was set-up in front of the aquarium and to record the activation and an acoustic receiving system (MAP_RTA, Lotek Wireless Inc.) was employed to monitor acoustic transmissions.

Implanted Group. One hour before the activation time, the 5 group B tags were removed from the "simulated peritoneum". Five freshly killed rainbow trout were obtained from a local fish market, each weighing approximately 1.5 lbs. A 1.5 cm incision was made in the mid-ventral surface of the fish stopping anterior to the pelvic girdle and the tag was inserted into the peritoneal cavity. The incision was closed with 3 or 4 knotted sutures using 2-0 silk. An identification tag was attached to the dorsal fin of each fish with the ID number of the implanted tag printed clearly on each. The implanted and tagged fish were then placed in an aquarium. Again, a video recording was made and a MAP_RTA receiver was used to monitor the unique ID transmissions from each tag within the group.

Results

All tags from both groups produced identifiable acoustic transmissions throughout the 14 day test period. Similarly, all tags activated within 2 minutes of their specified 14-day timepoint. There was no leakage of acetic acid from the tags, however, some tags had incomplete mixing of acetic acid and $NaHCO_3$, resulting in only partial bladder inflation. Table 4 provides detailed results from each tag related to the inflation of the tag and expulsion of KCl (Group A) and

flotation of the fish (Group B) following activation. For illustrative purposes, figure 6 shows before/after for fish B9 and B10.

Figure 6. Final Tests of Implanted Tags B9 and B10. Photos show fish prior to and after activation and flotation of fish at day 14.

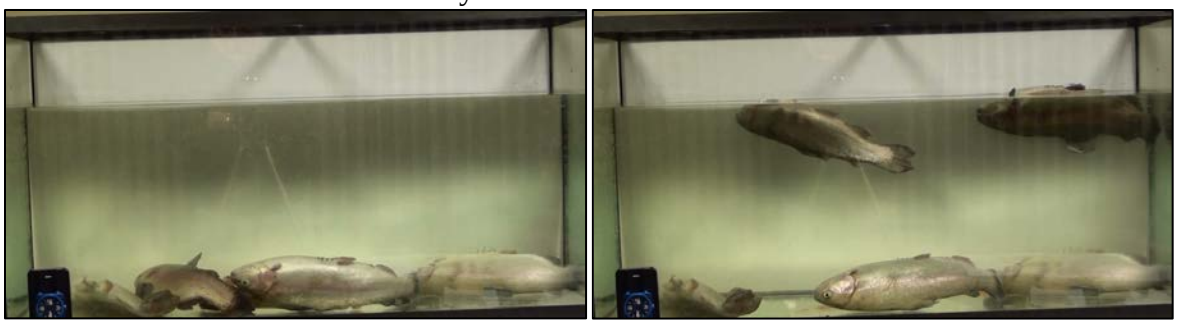


Table 4. Description of Each Tag at Day 14 Activation Time

Tag	Activation	Inflation Mechanism	KCl Discharge
A1	Squib activation at prescribed time; forceful detonation	Rapid, full bladder inflation and ascent to the surface	Yes
A2	Squib activation at prescribed time; muted detonation	Slight bladder inflation	Yes
A3	Squib activation at prescribed time; moderate detonation	Slight bladder inflation and ascent to the surface	No
A4	Squib activation at prescribed time; weak detonation	Slight bladder inflation	No
A5	Squib activation at prescribed time; forceful detonation	Partial bladder inflation and ascent to the surface	Yes
B6	Squib activation at prescribed time; moderate detonation	Slight increase in fish buoyancy	Yes
B7	Squib activation at prescribed time; inaudible detonation	No change in fish buoyancy	No
B8	Squib activation at prescribed time; inaudible detonation	No change in fish buoyancy	Yes
B9	Squib activation at prescribed time; loud detonation	Rapid ascent and rapid inflation	Yes
B10	Squib activation at prescribed time; loud detonation	Rapid ascent and rapid inflation	Yes

The root cause of the inconsistent bladder inflation and payload release is failure of the glass ampule to fracture following squib detonation. The evidence suggests one or more related causes:

1) Inconsistency in squib detonation force
2) Strengthening of vial through $NaHCO_3$ packing or cementing (possibly a time dependent process resulting from high humidity)
3) Separation of the detonated squib from the wall of the vial or, alternatively, strengthening of the vial from gluing the squib to the vial surface

Modifications to improve the reliability of the tags would focus on refining the inflation mechanism to more effectively fracture the vial. Squib detonation force and the magnitude and rate of $NaHCO_3$/acetic acid mixing need to be consistent and sufficient to fully mix reagents and inflate the bladder. Potential avenues that could be explored include modestly increasing the size of the squib; structural modifications to ensure squib force is directed toward the ampule; and lessening the protective effects of $NaHCO_3$ packing or cementing (e.g, use of coarse crystalline $NaHCO_3$ or including a desiccant).

Task 4 Summary

The final testing verified that the essential components of the fish tracking/recovery technology are in place, but the flotation mechanism is not consistently reliable. Table 5 presents the summary of the final testing. The root cause of the inconsistent inflation is known; possible design modifications to rectify the problem have been explored.

Table 5. Summary of Tag Performance at Final Testing

Endpoint	No. of Tags (out of 10)
Acoustic Transmission	10
Time Specific Activation	10
Full Inflation of bladder	3
Release of KCl	7

Because testing had to move to an ex-vivo format, the capability of the tag to rapidly euthanize has not been tested (release of KCl is the closest endpoint). Prototype tags establish proof of concept that tags can be developed with sufficient power, tracking capabilities, timed activation, and coupled flotation/euthanization agent release mechanisms. However, ultimately, project objectives were not achieved as developed tags did not meet the go/no go criterion.

CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

Substantial progress was made toward developing a reliable and functioning tag design capable of achieving design requirements. However, the go/no go criterion was not achieved. The current version incorporates sufficient power, a fully functioning tracking capability, and activates reliably. These functions have been integrated into a design that is biocompatible and is well tolerated by fish. The cause of premature death experienced in early in-vivo testing has been identified, isolated, and fixed. The flotation mechanism is not yet consistently reliable and the euthanization mechanism (KCl discharge) is reliant on the flotation mechanism to function. The cause of the inconsistent inflation has been isolated (lack of acetic acid vial breakage) and an understanding of the factors that inhibit vial breakage is beginning to emerge (see discussion above). At this point in the project, time and funds had run out to perform additional refinements and testing.

To achieve the limited scope objectives, the flotation and euthanization mechanisms need to be verified. This verification would entail ex-vivo testing of design modifications that increase the extent and rate of mixing reactants and reagents for gas generation. For example, the squib size and placement could be modified to increase the mixing force; granular $NaHCO_3$ could replace the current powdered form to increase air spaces and decrease packing, small

amounts of a desiccant could be included to decrease possible cementing. The overall reliability and effectiveness of the tag to activate at a specified time, euthanize fish, and float them to the surface would be tested per the original plan (implanting 10 fish, slated to activate at 30 days, with a success criterion of 50% recovery of fish at the surface at the specified time point).

At this point, two primary uncertainties remain. *First, can mixing of the acetic acid and $NaHCO_3$ be achieved to generate gas?* Certainly it is well understood that mixing the reagents reliably produces gas. Squib detonation, intended to break the glass ampule of acetic acid, also occurs with great reliability. The physical breakage of the vial is believed to be inhibited by an as-of-yet unkown, but likely simple cause; an assertion supported by the repeatability of vial breakage by the squib under controlled conditions. Thus, it is believed that this is a manageable problem that can be rectified with minor design modifications.

Second, will the KCl release via the inflation mechanism effectively euthanize the fish? We know from testing that KCl needs to enter the bloodstream to elicit rapid euthanization. Forceful expansion of the membrane inside the fish is anticipated to cause capillary breakage, permitting the IV entry of discharged KCl. However, as described above, this has not yet been demonstrated. KCl is a good choice as a toxicant because of its otherwise benign attributes and low potential for unintended adverse effects if consumed or contacted by fishers or wildlife. However, small quantities of other well-known fish euthanization agents, such as benzocaine hydrochloride, could also be readily incorporated into the tag design.

In summary, while the go/no go criterion was not demonstrated, substantial progress has been made towards developing a functioning tag. Prototype tags establish proof of concept that tags can be developed with sufficient power, tracking capabilities, timed activation, and coupled flotation/euthanization agent release mechanisms. However, issues remain with the reliability of the flotation mechanism and euthanization has not been proven. It is anticipated that additional design modification and testing could address remaining uncertainties to achieve the project objectives and go/no go criterion.

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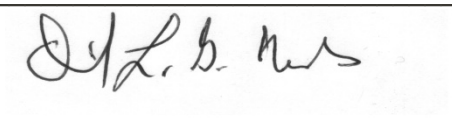
APPENDIX 1. OREGON STATE UNIVERSITY'S APPROVED ANIMAL CARE AND USE PROPOSAL

PI Assurance and Funding Information

ACUP ID (IACUC OFFICE ASSIGNED NUMBER):

1. Principal Investigator (PI): David L. G. Noakes
   (Students may not serve as PIs)
2. Department: Fisheries and Wildlife Department

3. Assurance: I accept and assure compliance with all federal, state and institutional regulations concerning the care and use of animals. I will notify the Institutional Animal Care and Use Committee of any changes in the proposed project or personnel prior to initiating or continuing any animal use and will report any adverse events. I certify that all laboratory personnel handling animals will be adequately trained for all animal protocols used in the project and will be enrolled in the Institutional Occupational Health Program for Personnel with Animal Contact. I attest that the information contained in this ACUP submission is complete and accurate to the best of my knowledge.



Principal Investigator signature
Date 28 November 2010

4. Project title or course name and number: Development of an archival fish tag

5. Is this a project continuation from an expired/expiring ACUP?: **Yes**☐ **NoX**
   If yes, prior ACUP #

6. Funding source(s) list all that apply, including associated titles: Internal (OHRC); USGS; Department of Defense, Strategic Environmental Research and Development Program (SERDP): Lotek Wireless (http://www.lotek.com/)

Provide a copy of the grant proposal or class syllabus. An electronic version is preferred.

Justification for Use of Live Vertebrates

7. In lay terms, explain the objectives or goals and benefits or significance of the proposed research, testing, or instructional use of animals. This section should be drafted so a non-scientist would understand it. If technical terms or scientific jargon need to be used, please provide explanations the non-scientist will understand.

We are working with industrial and federal government partners to develop an achival ultrasonic tag to be used in freshwater fishes. Overall, the project is intended to develop a replacement strategy for electroshocking and collection of native fishes for chemical analysis in bioaccumulation studies. Those efforts suffer from an inability to fully understand the areas in which a fish has resided, fed,

and undergone contaminant exposures. Collected fishes will also vary in size, sex, and condition which increases variability in their contaminant concentrations. Hence, intensive sampling and large numbers of fish are necessary to develop a robust relationship between contaminant exposures in sediments and the levels in fish. We seek to develop a fish tracking/recovery technology that monitors fish location over time and permits recovery of the fish at specified time points. Such an approach would eliminate the afore-mentioned uncertainties and ultimately reduce the number of fish that need to be sacrificed to characterize the bioacculuation of contaminants in fish. At this point, a device design has been developed, but it is untested. The tag design includes an internal acoustic transmitter for tracking and ability to "activate" the tag at a specified time. Activation is designed to remotely euthanize fish by percussive stunning (from squib detonation) and chemical intoxication; and permit surface recovery via gas/foam generation within a membrane.

Four steps are envisioned:

Step 1: Establish lethal dose of potassium chloride (KCl)

Step 2: Establish reliability of device activation and integrity of implantation site to balloon deployment

Step 3: Refine device by evaluating reliability (activation) and effectiveness (euthanasia and surface recovery) of KCl-loaded prototype devices.

Step 4: Full-scale final device testing

The first step in the archival tage development is to test an alternative method for euthanasia of fishes that could be used under field and laboratory conditions. MS-222 is the standard anesthetic agent for fishes. Anesthetized fishes can then be euthanized by one of several acceptable techniques, including injection of a saturated solution of potassium chloride (AVMA 2007). However, the AVMA guidelines are based upon intravenous or intracardiac injection, and the dosages required are given only in general terms for all species. Intravenous or intracardiac injection of any agent in fishes is feasible only for large individuals, and practical only under controlled laboratory situations. The realistic alternative method of administering the solution is by injection into the body cavity (intraperitoneal). This procedure is feasible for fishes of any size, under any conditions. In this project we will administer the potassium chloride intraperintoneally to fish that have been anesthetized with MS-222 under controlled laboratory conditions. The potassium chloride will be rapidly taken up from the peritoneal fluid into the circulatory system. We will measure the time to death (cessation of opercular respiratory movements), and from that we will determine the effective lethal dose of potassium chloride as a function of size (live body mass) of the fish. We will use rainbow trout (*Oncorhynchus mykiss*) as our study species, as it is widely used in field and laboratory studies, and it is representative of other salmon and steelhead that are very commonly studied in the Pacific Northwest. The results of this study will provide accurate guidelines for the use of intraperitoneal injeciton of potassium chloride as an acceptable method for euthanasia in fishes.

At the same time as we are establishing the effective dose for intraperitoneal injection of potassium chloride, we will initiate the second step of the project. This will be to test the placement of prototype versions of the archival tag into the body cavity of rainbow trout, and to verify that the tags will deploy as programmed (set by an internal timer at the time of surgery) and that the tags will not be expelled from the implantation wound (incision) when the membrane inflates. These prototype tags will not contain KCl as this effort is intended to evaluate the reliability and effect of the deployment mechanism. The prototype tags will be implanted surgically into the body cavity of a

small number of rainbow trout, using routine, accepted procedures (numerous other OSU ACUPs have approved these procedures). Just prior to timed deployment of the tags the trout will be euthanized with MS-222, and the fish will be held under close observation for tag deployment following the death of the fish. This will establish that the tags deploy as designed, and will establish the length of time after surgery necessary before tag deployment.

The third step of the project is contingent upon successful completion of the first two requirements. Once we have established the effective dose for potassium chloride, and have determined that the tags will deploy successfully and without re-opening the implantation site (incision), we will test the effectiveness of KCl-loaded tags implanted into rainbow trout that will be held in large observation tanks. The trout will be observed in these captive conditions to determine if the tags deploy and operate as designed.

The final step is to verify the reliability and effectiveness of the developed tag in a longer deployment that more realistically simulates field conditions.

Completion of these steps would result in the tag manufacturers producing the archival tags on a commercial basis for use in field studies.

8. Provide an explanation for why live vertebrate animals are needed for the proposed project. In addition, please describe the non-animal alternatives that were considered.

Numerous fish species are collected and analyzed to evaluate bioaccumulation of contaminants in aquatic systems. Fish are the most relevant endpoint, because they are consumed by humans and other predators. The technology developed herein is intended to be used in limited numbers of hatchery raised fish, in lieu of collection of native fish, so fish are still required. Initial iterations of device testing and refinement are currently being conducted ex-vivo to minimize the numbers of animals that will be needed for device testing and refinement.

The project is to determine the precise dose levels of potassium chloride required for euthanasia in fishes, so live fish must be used as the study species. We must test for the deployment of tags in euthanized fish, to determine the function of the tag system in a fish, rather than a non-animal alternative. Finally, the tags must be tested in live fish as that is the end purpose of the development program.

9. Explain why each specific species was selected. What biological characteristics make this the best model for the work?

Rainbow trout is our study species because it is readily available in large numbers, at a range of sizes at any time of the year. It is widely used as a representative “cold water” fish species for many studies of physiology, endocrinology, behavior and ecology and there is a very extensive literature on the biology of this species. It is also generally tolerant of holding and handling procedures. We have the species available under stock holding ACUPs at the Fish Genetics and Performance Laboratory and the Oregon Hatchery Research Center. It is closely related to other salmon and steelhead species and so results can be extrapolated to those species. Rainbow trout has been selected because it is similar morphologically to the types of fishes that are typically collected in fish sampling to support

contaminant bioaccumulation assessments. The targets of these bioaccumulation studies are typically adult fish that are fished and consumed at contaminated sites. The rainbow trout is considered a good surrogate for most freshwater fishes.

Animal Species and Use Categories

10. LIST THE SPECIES AND NUMBERS OF ANIMALS THAT WILL BE USED BY CATEGORY BELOW. THE CATEGORY INDICATED IS DETERMINED BY THE PROCEDURES TO BE PERFORMED ON THE ANIMALS. A DESCRIPTION OF EACH CATEGORY, AS DEFINED BY THE USDA'S ANIMAL WELFARE ACT, IS PROVIDED BELOW.

Add additional lines or delete unused lines as needed.

Common name of species	B	C	D	E
Rainbow trout			72	

"B" Breeding Colony or Holding Protocols

"C" No more than momentary or slight pain or distress and no need for pain-relieving drugs, or no pain or distress.

"D" More than momentary discomfort, distress, or pain in which a veterinarian or human doctor would require relieving this with anesthetics, analgesics and/or tranquilizer drugs or other methods for relieving discomfort, distress, or pain.

"E" More than momentary discomfort, distress, or pain in which a veterinary or human doctor would require relief, however, the use of these agents would interfere with the scientific outcome.

11. Specify the number of animals needed and include any calculations used to determine minimum group or sample size. Also, include the "Animal Use Category" that corresponds to the proposed work. It is suggested that tables or charts be used so that experimental groups are clear and can be matched to procedures performed. An explanation of the procedures should not be included in this section.

As described in Sections 7 and 19, testing will occur in four steps:

In Step 1, we will use a maximum total of 20 rainbow trout. We will start by administering a volume (to be determined based on the size of the fish) of saturated KCl solution in water or a mass of crystaline

KCl (volume of KCl solution and crystaline material calculated from AVMA Guidelines to provide a lethal dose) to two fish each (4 fish total). If the fish die immediately after the KCl injection, then we will administer ½ that amount to two further fish for the wet and dry preparations, and so forth to identify a minimum effective dose resulting in euthanasia (4 fish total). With 2 fish each for dry and wet preparations this will allow for 5 iterations to identify the effective dose (20 fish total). Of course, that many may not be necessary. This effort has two objectives:

a) determine the effective volume of potassium chloride for euthanasia

b) determine the effective dose of potassium chloride in relation to body size for the size range of trout likely to be used in research.

To achieve the first objective we will use trout of intermediate size (100 grams live body mass). The AVMA Guidelines for Euthanasia recommend rapid intravenous or intracardiac injection of 1 – 2 mmol . kg^{-1} of saturated potassium chloride solution. We will use intraperintoneal injection of liquid and crystaline KCl, and so we have to administer a range of five injection volumes, in two fish at each volume for liquid and crystaline KCl . To achieve the second objective will inject the effective dose (mmol . kg^{-1}) determined from the first series of injections into fish of the different sizes (2 fish at five size classes live body mass; for a total of 20 fish).

In Step 2, we will surgically implant prototype tags without KCl into 6 large rainbow trout (3 timepoints post-implantation will be tested on 2 fish each).

In Step 3, we will test the KCl-loaded devices on 4 trout to ascertain reliability (devices activate) and effectiveness (devices cause rapid mortality). Up to 4 iterations will be tested during this step if initial device designs are not adequate. (Up to 16 large rainbow trout will be tested).

In the Step 4, we will surgically implant tags into 10 large rainbow trout.

Rationale for animal numbers:

Steps 1 and 2: In considering the necessary number of animals, we decided that results from two fish at each concentration would provide a reasonable level of confidence with regard to the repeatability of device performance and toxicity of pottasium chloride while not uneccesarily sacrifing a greater numbers of test animals.

Step 3: Device testing and refinement requires greater resolution as to the reliability and effectiveness of the newly developed device because the device will then be submitted to final go/no go testing. Tests using 4 individuals have been selected to provide greater certainty without uneccesarily sacrifing greater numbers of animals.

Step 4: Final Device Testing: A longer deployment period similar to anticipated field conditions is envisioned for the final device test. This test will represent the final evaluation of the device prior to determining whether to pursue or forego further device developent (go/ no go testing). As such, the test should provide greater confidence in the efficiacy and reliability of the final device design, or greater resolution as to its failure rate. One test of 10 fish has been chosen to provide greater certainty without uneccesarily sacrifing greater numbers of test animals.

These are the minimum numbers (72 fish total) of fish to test for effective dose, to test for the relationship between dose and body mass, and to test for tag deployment reliability and euthanasia effectiveness. In all cases these tests are "all or none" (i.e., either the dose is effective or not, the tags deploy or not) so we can minimize sample sizes.

12. Provide a narrative description of the methods and sources that were used in consideration of alternatives to the use of animals and alternatives to painful procedures. An alternative is any procedure that results in a Reduction in the number of animals used, Refinement of techniques (less painful or invasive), or Replacement of animals with non-animal models (the 3 R's). The minimal narrative should include: the sources and/or databases searched (i.e., index Medicus, Medline, Current Research Information Services, Animal Welfare Information Center), the date of the search, years covered by the search, and the key words and/or search strategy used to determine that no alternatives were available to the painful or distressful procedure. The IACUC Guideline for Literature Searches: http://oregonstate.edu/research/ori/animal/lit_search.html.

The current project is quite unique in scope as it requires the remote euthanization of fish. The proposed technique requires coincident euthanization and an increase in fish buoyancy for surface recovery. Numerous publications were consulted and literature searches were conducted to investigate currently practiced and accepted humane methods of fish immobilization and dispatch to identify methods that could be adapted to effect remote euthanization of a fish. As all methods identified required the physical contact with the target animal to carry out, none found was suitable to the current application. As it was determined that the remote dispatch of a fish could be technologically accomplished by physical means, it was decided to rely upon the trauma that the buoyancy increasing apparatus would cause to stun the fish. Chemical agent(s) that could be released at the time of device activation to increase the speed of onset of death and minimize any attendant suffering were also explored, with KCl identified as the euthanizing agent and heparin added to maximize uptake.

The first step of this project is to determine the effective lethal dose for intraperitoneal injection of potassium chloride in fishes, so no alternative to live animals is possible. The remaining steps require the use of live fish to test for tag deployment, so no alternative is possible.

For Steps 1 and 2, the number of animals to be used is the minimum number requried to produce credible results (2 animals at each dose, deployment time, or body size). The fish will be anesthetized (Stage 5) at the level normally used for invasive surgery or other procedures, so they will be insenstive to any pain or discomfort.

For the remaining steps we will use standard procedures for fish surgery and tag implantation – techniques that we have helped established and have utilized in a number of fish species over many years.

We searched online (18 November 2010), using Google Scholar, Index Medicus and Web of Science, with no limits on dates, using all combinations of the terms: AVMA, euthanasia, fish, KCl, potassium chloride. For the second and third steps we searched for combinations of: fish, trout, salmon, surgery, tag, implanting, tagging, acoustic tag, radio tag, telemetry. As there is a very large number of such references on these topics we have cited only the major references, particularly ones that include earlier literature.

Relatively few references were found in this search for potassium chloride as a secondary means of euthanasia in fishes; all are listed below (References, with references contained therein). The following text is the most specific, and the most relevant to our proposal. It is quoted from the AVMA Guidelines on Euthanasia (Formerly the Report of the AVMA Panel on Euthanasia) (2007):

"**POTASSIUM CHLORIDE IN CONJUNCTION WITH PRIOR GENERAL ANESTHESIA**
Although unacceptable and condemned when used in unanaesthetized animals, the use of a supersaturated solution of potassium chloride injected intravenously or intracardially in an animal under general anesthesia is an acceptable method to produce cardiac arrest and death. The potassium ion is cardiotoxic, and rapid intravenous or intracardiac administration of 1 to 2 mmol/kg of body weight will cause cardiac arrest. This is a preferred injectable technique for euthanasia of livestock or wildlife species to reduce the risk of toxicosis for predators or scavengers in situations where carcasses of euthanatized animals may be consumed.
Advantages—(1) Potassium chloride is not a controlled substance. It is easily acquired, transported, and mixed in the field. (2) Potassium chloride, when used with appropriate methods to render an animal unconscious, results in a carcass that is potentially less toxic for scavengers and predators in cases where carcass disposal is impossible or impractical.
Disadvantage—Rippling of muscle tissue and clonic spasms may occur on or shortly after injection.
Recommendations—It is of utmost importance that personnel performing this technique are trained and knowledgeable in anesthetic techniques, and are competent in assessing anesthetic depth appropriate for administration of potassium chloride intravenously. Administration of potassium chloride intravenously requires animals to be in a surgical plane of anesthesia characterized by loss of consciousness, loss of reflex muscle response, and loss of response to noxious stimuli. Saturated potassium chloride solutions are effective in causing cardiac arrest following rapid intracardiac or intravenous injection. Residual tissue concentrations of general anesthetics after anesthetic induction have not been documented. Whereas no scavenger toxicoses have been reported with potassium chloride in combination with a general anesthetic, proper carcass disposal should always be attempted to prevent possible toxicosis by consumption of a carcass contaminated with general anesthetics."

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13. Does this proposal duplicate a previous use of animals in teaching or research? **Yes☐ NoX**
If yes, why is duplication necessary (e.g., new group of students, more rigorous study design, new information, components that were not evaluated previously, etc)?

Animal Care

14. Animal housing location: List building, floor, room, barn, corral, paddock, or pasture as applicable. Please note that if this is a new housing area, approval from the OSU Attending Veterinarian and IACUC will be needed prior to housing animals.

Fish Genetics and Performance Laboratory ("Smith Farm"), Highway # 34, Corvallis

15. Indicate the source providing the animals (e.g., Simonsen Labs, etc.)

Fish Genetics and Performance Laboratory or Oregon Department of Fish and Wildlife (OHRC)

16. Who will provide husbandry care for the animals? List unit or person's name.

Rob Chitwood, USGS Fish Coop Research Unit, Fisheries and Wildlife Department, OSU

Kristen Berenkamp

Sierra Lewis

Julia Unrein

17. Who will provide veterinary care for sick or injured animals? If someone other than the OSU Attending Veterinarian will provide this care, please list the individual, their relevant credentials, and their contact information. The OSU Attending Veterinarian has the ultimate responsibility for the veterinary care of all animals used for teaching, research, and testing at Oregon State University. Others may provide veterinary care, with the approval of the University Attending Veterinarian and IACUC.

Oregon Department of Fish and Wildlife fish pathologist Tony Amandi. If medications need to be administered, then the OSU Attending Veterinarian (or individual delegated by the AV) will be contacted in the case of any injured or sick trout.

ANIMAL USE

18. Identify all areas where animal procedures will be performed. If procedures will be performed in housing areas, please include these in the list. The information should include: building, room or any other relevant description.

Fish Genetics and Performance Laboratory

19. Describe in detail all procedures involving the animal(s). This section should include sufficient description of the experimental design, such that it is clear which animals, or groups of animals, will undergo which procedures. Include doses and routes of administration for all experimental agents, medications, chemicals, etc. Describe the order in which procedures will be performed, including the endpoints of the study. There should be direct correspondence between the descriptions of groups in the "Justification for Use of Live Vertebrates" section, Questions 11 and 12. Do not copy and paste grant information into this section.

The overall objective of this project is: 1) a working acoustic device that, 2) at a programmed time point causes lethality and, 3) permits surface recovery.

Four steps are envisioned:
Step 1: Establish lethal intraperitoneal dose of KCl
Step 2: Establish reliability of device activation and integrity of implantation site to balloon deployment
Step 3: Refine device by evaluating reliability (activation) and effectiveness (euthanasia and surface recovery) of KCl-loaded prototype device.
Step 4: Full-scale final device testing

Step 1: Fish will be obtained from the stock of rainbow trout held by the Oregon Department of Fish and Wildlife (OHRC) or the FPGL (Stock Holding ACUPs) as required. Researchers will work in groups of two people to capture individual trout from the stock tank, place them into a bucket filled with aerated water from the stock tank and transfer them to the anesthesia aquarium. Fish will be selected by size, based on the protocol for testing. The effective dose of potassium chloride will be determined by injecting different volumes of saturated potassium chloride and different masses of crystalline KCl into fish of the same size. The effective dose in relation to body size (mmol. kg^{-1}) will be determined by injecting the volume determined by this procedure into trout of four additional size ranges, in proportion to their body mass.

A stock solution of anesthetic (MS-222, 150 mg. L^{-1}; buffered to pH 7.0 with sodium bicarbonate) prepared and kept aerated by compressed air dispersed through air stones will be used to anesthetize test fish to Stage 5 anesthesia (fish unresponsive to external stimuli, and unable to maintain equilibrium but with persistent ventilatory opercular movements) (Keene et al. 1998). Potassium chloride will be prepared as a saturated

solution in deionized water. Individual doses of the saturated potassium chloride solution will be drawn into a sterile syringe, fitted with a sterile needle for each trout. Premeasured does of crystalline KCl will be administered via a syringe needle using a trochar.

Prior to KCl injection, individual rainbow trout will be immersed in the aerated MS-222 anesthetic and observed until they reach Stage 5 anesthesia (about 2 minutes; Keene et al 1998). One researcher will observe the fish; a second researcher will record time of transfer to the aquarium and time to each stage of anesthesia (Keene et al. 1998). As soon as the trout reaches Stage 5 anesthesia it will be removed from the aquarium with a dip net. One researcher will hold the fish in the dip net while the second researcher injects the specified volume of potassium chloride solution or crystalline KCl into the peritoneal cavity. The trout will then be immediately placed back into the observation container and observed for cessation of ventilatory opercular movements. Again, one researcher will observe the fish, the other will record times. Fish will observed for at least 10 minutes after cessation of ventilatory opercular movements. We expect from published information (AVMA 2007) that the potassium chloride will cause cessation of ventilatory opercular movements within 1minute after injection. Any trout that does not show cessation of ventilatory opercular movements within 5 minutes after potassium chloride injection will be euthanized by immersion into a lethal concentration (350 mg. L^{-1}, buffered to pH 7.0 with sodium bicarbonate) of MS-222 in a separate glass aquarium. Trout will be held in that lethal concentration for at least 10 minutes after cessation of ventilatory opercular movements. All trout that have been observed for at least 10 minutes after cessation of ventilatory opercular movements will be removed from the glass aquarium by dip net. Each trout will be decapitated with a large knife, and the head and body wrapped in paper towels and placed into a freezer (-20 ^{O}C). At the end of all the trials all frozen carcasses will be disposed of in the normal domestic waste stream.

Data recorded from the observations will be the times to induction of Stage 5 anesthesia after immersion in the MS-222 solution (150 mg. L^{-1}, buffered to pH 7.0 with sodium bicarbonate), time to cessation of ventilatory opercular movements after injection of potassium chloride, and time to cessation of ventilatory opercular movements after immersion in the lethal dose (350 mg. L^{-1}) of MS-222. These data will be for observations of trout of the same size (20 grams live body mass) injected with a range of volumes of saturated potassium chloride (two trout at each dose) and for trout of a range of sizes (10 - 1000 grams live body mass; two trout at each size) for the injection of the effective dose (mmol. kg^{-1}) of potassium chloride.

Steps 2 - 4 involve surgical implantation of prototype tags that will follow standard surgical procedures used in our laboratories for previous ACUP projects. Fish will be anesthetized by immersion into an aerated solution of anesthetic (MS-222, 150 mg. L^{-1}, buffered to pH 7.0 with sodium bicarbonate) in a darkened container until they reach Stage 5 anesthesia (Keene et al. 1998). Fish will then placed supine (ventral side up) in a plastic covered foam wedge to hold them securely. Commercially available “Stress Coat” will be applied to the wedge to minimize scale loss. The gills will be continuously perfused with an anesthetic solution (MS-222, 150 mg. L^{-1}, buffered to pH 7.0 with

sodium bicarbonate) dripped through a perforated tube placed in the buccal cavity to maintain Stage 5 anesthesia. A small midventral incision will be made between the pectoral and pelvic fins with a sterile scalpel blade. The prototype tag will then pushed gently into the body cavity and the incision will be sutured (surgical monofilament, three simple interrupted sutures). The area surrounding the sutures will be coated with surgical glue (VetBond) to minimize fluid exchange through the incision. Fish will be allowed to recover from surgery in a container of aerated fresh water until they have reached Stage 1 recovery (Keene et al. 1998). They will then be placed into individual fiberglass tanks (1 meter diameter) for routine holding, care and maintenance according to standard procedures at the FGPL.

Step 2 includes the placement of prototype versions of the archival tag into the body cavity of rainbow trout, and to verify that the tags will deploy as programmed (set by an internal timer at the time of surgery) and that the implantation site has healed sufficiently such that it will not reopen upon deployment of the tags internal balloon. The prototype tags will be implanted surgically into the body cavity of a small number (6) of rainbow trout, using routine, accepted procedures (numerous other OSU ACUPs have approved these procedures). Fish will be alowed to recover from surgery as described above, and held under routine maintenance and care conditions at the FGPL. Just prior to timed activation of the tags (7, 10, 13 days post surgery) the trout will be euthanized with MS-222 (350 mg. L^{-1}, buffered to pH 7.0 with sodium bicarbonate) in a darkened container for at least 10 minutes after the cessation of ventilatory opercular movements (respiration). The fish will then be held under close observation for tag deployment following the death of the fish (tag deployment within 1 hour after death of the fish).

Step 3 is contingent upon successful completion of the first two steps. Once we have established the effective dose for potassium chloride, and have determined that the tags will activate successfully without reopening the implantation incision (sutures), we will test the effectiveness of tags loaded with KCl in 4 rainbow trout. These tests build upon the earlier findings and evaluate the device reliability (i.e., 100% of devices activate) and effectiveness (i.e. the device causes rapid euthanasia and permits surface recovery of the fish). A smaller number of animals and shorter time period (as determined in Step 2) will be used so that device refinements can be made if necessary prior to full-scale testing. These 4 fish will be held individually in large (2 meter diameter, 1 meter deep) fiberglass tanks for this test. These fish will be observed continuously by two people (one to record observations, the other to record times) beginning 1 hour before the timed activation of the tag. Device activation will result in percussive stunning, expansion of a balloon in the intraperitoneal cavity, and release of powdered potassium chloride (KCl) and heparin. The stunning and lethal dose of KCl will result in a rapid death with minimal pain perception. The lethal dose of KCl will be established in the first step of the project (see above). It is anticipated that percussion and expansion of the balloon will result in capillary breakage that facilitates uptake of KCl. Heparin is included in the prototype tag to decrease clotting and further enhance uptake of KCl into the bloodstream. Following device activation, fish will be monitored for euthanasia and surface recovery. If euthanasia, as determined by cessation of gill ventilation, does not occur within 5 minutes, the fish will be removed from the tank and euthanized by immersion into a lethal concentration (350 mg. L^{-1}, buffered to pH 7.0 with sodium bicarbonate) of MS-222.

Full-scale testing. Following device refinement and a determination that the device is both effective and reliable, a similar testing strategy will be used, over a time-frame similar to actual field implementation. Fish will be held in large observations tanks (2 meter diameter, 1 meter deep fibreglass tanks). The tags will be timed to deploy at 30 days. These fish will be observed continuously by two people (one to record observations, the other to record times) beginning 1 hour before the timed activation of the tag. The trout will be observed to determine if the tags deploy and operate as designed at that time. If the tags fail to deploy as designed, the fish will be euthanized with MS-222 (350 mg. L^{-1}, buffered to pH 7.0 with sodium bicarbonate) in a darkened container for at least 10 minutes after the cessation of ventilatory opercular movements (respiration).

20. Describe method(s) of euthanasia and carcass disposal or other disposition, if applicable. Methods of euthanasia employed should be acceptable, according to the American Veterinary Medical Association (AVMA) "Guidelines on Euthanasia" (2007). If a method is considered conditionally acceptable or unacceptable, scientific justification is required. Methods utilized to ensure euthanasia (i.e., secondary means) also should be included in this description.

Step one – determination of effective dose of potassium chloride as a method of euthanasia. All fish will be anesthetized and held at Stage 5 anesthesia, with MS-222, (150 mg. L^{-1}, buffered to pH 7.0 with sodium bicarbonate). Death will be judged by cessation of opercular (ventilatory) movements for a period of at least 10 minutes. Any fish that do not exhibit cessation of opercular movements within 5 minutes of injection of the potassium chloride will immediately be euthanized by immersion in a solution of MS-222 (350 mg. L^{-1}, buffered to pH 7.0 with sodium bicarbonate,) until opercular movements have ceased for at least 10 minutes.

Step two and step three – fish will be euthanized by immersion in a lethal dose of anesthetic in a darkened container (MS-222; 350 mg. L^{-1}; buffered to pH 7.0 with sodium bicarbonate) for at least 10 minutes after cessation of ventilatory opercular movements (respiration).

The secondary method of euthanasia will be decapitation with a large knife.

Carcasses will be frozen (-20 ^{O}C) and then disposed in the domestic waste stream.

21. Describe any expected adverse consequences that may occur to the animal. These can include complications from the procedures performed, loss of life (death), adverse phenotypes from transgenic strains, surgical complications, etc.

No adverse consequences expected. These fish are held routinely at the Fish Genetics and Performance Laboratory. The administration of Stage 5 anesthesia with MS-222 is a standard operating procedure for this species. The surgical procedures are also standard for the FPGL.

21A. Describe any actions that will be taken to prevent or minimize deleterious consequences.

All persons involved in this project have completed the required training, and all have laboratory and field experience with holding, handling and surgical procedures on salmonid fishes. All handling procedures and administration of MS-222 anesthesia are standard operating procedures at the FPGL. All procedures will be carried out by people working in teams of two individuals. All fish will be checked daily as part of the routine care and maintenance activities at the FPGL.

22. Check any of the following that apply. Complete and submit all applicable supplements.

☐ Antibody production (Antibody Production Supplement)
☐ Breeding colony or sentinel animals (Breeding Colony & Sentinel Animal supplement)
☐ Death of the animals as an indicator or data point in the research or instruction (Death as an End Point Supplement)
☐ Field studies (Field Studies Supplement)
X Surgery (Surgery Supplement)
☐ Herd animals including poultry or stocks of fish

23. Other agents used in the research - check appropriate boxes and complete the Research Agent Use in Animals Form that can be found at: http://oregonstate.edu/ehs/forms/auf.doc It is the responsibility of the PI to secure any other compliance committee approvals necessary. Questions regarding approval process for other safety committees (Institutional Biosafety Committee (IBC), Radiation Safety Committee (RSC), and Chemical Safety Committee (CSC)) should be directed to the appropriate committee. The IACUC Office can provide this contact information.

☐ Biohazards administered to the animal
☐ Radioactive isotopes administered to the animal
☐ Infectious agents administered to the animal
☐ Carcinogens administered to the animal
☐ Recombinant DNA products administered to the animal
☐ Breeding or creation of genetically modified animals

Participants (P) Supplement

List all staff that will perform work described in this application. For classes, include instructional staff only. All Participants must complete Animal Welfare Education Training and complete the OHS required form.

Animal Welfare Education:
http://oregonstate.edu/research/ori/animal/ed.htm
http://oregonstate.edu/research/ori/animal/iacuc_ed.html#training
Occupational Health Services (OHS):
http://oregonstate.edu/research/ori/handler.htm
http://oregonstate.edu/occupationalhealth/

P1: In the event that an emergency issue arises, it is important that the University Veterinary staff or IACUC be able to contact an individual who can make decisions for animals described in this proposal. Please list, in preferred contact order, such individuals, their emergency contact information (i.e., cell phone, pager, home phone, etc.). The PI responsible for this project should be the first person listed.

P2: Add or remove additional personnel boxes, as needed

Name	David Noakes	**Department**	Fisheries & Wildlife
Work Address	104 Nash Hall, Oregon State University		
Work email	david.noakes@oregonstate.edu		
Work phone number	541-737-1953 First Contact in case of emergency Emergency Contact: cell phone: 541-908-1704; home phone: 541-758-9030		
Role (PI, laboratory staff, post-doc, student, etc.)	Co-PI		
Animal Welfare Education Completed (Y/N)	Y	**OHS Enrollment Completed (Y/N)**	Y
Experience and Qualifications	More than 30 years experience in field and laboratory situations with care and holding, capture and tagging of fishes		

Name	Carl B. Schreck	**Department**	Fisheries & Wildlife

Work Address	104 Nash Hall, Oregon State University		
Work email	carl.b.schreck@oregonstate.edu		
Work phone number	541-737-1961 Second contact in case of emergency Emergency contact: cell phone: 541-740-1021		
Role (PI, laboratory staff, post-doc, student, etc.)	Co-PI		
Animal Welfare Education Completed (Y/N)	Y	**OHS Enrollment Completed (Y/N)**	Y
Experience and Qualifications	More than 30 years laboratory and field experience with fish physiology, endocrinology and ecology using a variety of surgical techniques.		

Name	Julia Unrein	**Department**	Fisheries & Wildlife
Work Address	104 Nash Hall, Oregon State University		
Work email	Julia.unrein@oregonstate.edu		
Work phone number	541-737-1961		
Role (PI, laboratory staff, post-doc, student, etc.)	Laboratory staff		
Animal Welfare Education Completed (Y/N)	Y	**OHS Enrollment Completed (Y/N)**	Y
Experience and Qualifications	Extensive experience with holding, handling and maintenance of fish under laboratory conditions		

Name	Sierra Lewis	**Department**	Fisheries & Wildlife

Work Address	104 Nash Hall, Oregon State University		
Work email	sierra.lewis@oregonstate.edu		
Work phone number	541-499-1072		
Role (PI, laboratory staff, post-doc, student, etc.)	Postgraduate student		
Animal Welfare Education Completed (Y/N)	Y	**OHS Enrollment Completed (Y/N)**	Y
Experience and Qualifications	Several years experience in field and laboratory situations with care and holding, capture and tagging of fishes		

Name	Kristin Berenkamp	**Department**	Fisheries & Wildlife
Work Address	104 Nash Hall		
Work email	Kristin.berkenkamp@oregonstate.edu		
Work phone number	541-737-1961		
Role (PI, laboratory staff, post-doc, student, etc.)	Laboratory staff		
Animal Welfare Education Completed (Y/N)	Y	**OHS Enrollment Completed (Y/N)**	Y
Experience and Qualifications	Extensive experience holding, handling and maintaining fish in research laboratory		

Name	Rob Chitwood	**Department**	Fisheries & Wildlife
Work Address	104 Nash Hall, Oregon State University		
Work email	rob.chitwood@oregonstate.edu		
Work phone number	541-737-4008 First Contact in case of emergency Emergency Contact: cell phone: 541-602-0545		
Role (PI, laboratory staff, post-doc, student, etc.)	Facility Manager, FGPL		
Animal Welfare Education Completed (Y/N)	Y	**OHS Enrollment Completed (Y/N)**	Y
Experience and Qualifications	Many years experience in field and laboratory situations with care and holding, capture and tagging of fishes		

Surgery Supplement (S)
(Fill out only if applicable; one supplement for each Surgery type)

S1. Will this be a survival or non-survival (terminal) procedure? Please list the type (name) of the surgical procedure?

Survival

Surgical implant of acoustic transmitter tag into body cavity (intraperitoneal) of rainbow trout

S1a. If this is a survival surgery, is this a minor or major procedure? A major surgery is defined as one that penetrates or exposes a body cavity or produces substantial impairment of physical or physiological function.

Major surgery – midventral incision to insert tags, closing incision with sutures

S2. If the surgery involves recovery from general anesthesia (survival), will more than one survival surgery be performed on the same animal during its lifetime?
Yes☐ NoX
If yes, what is the timeframe between surgeries and the scientific justification for multiple survival surgeries on the same animal?

S3. Where will the procedure be performed? List the building, floor and room number or field site, as appropriate.

Fish Performance and Genetics Laboratory (Smith Farm)

S4. Describe pre-operative preparation of the animal, including; preparation of the surgical location, and surgeon. Include any anesthetic and analgesic agents (list name(s), dose(s) and route(s) of administration).

Surgical implantation of prototype tags will follow standard procedures. Individual fish will be anesthetized by immersion in an aerated solution of anesthetic (MS-222, 150 mg. L^{-1}, buffered to pH 7.0 with sodium bicarbonate) in a darkened container until they reach Stage 5 anesthesia (Keene et al. 1998). The person conducting the surgery will wash her/his hands twice with disinfectant soap, dry them with a sterile towel, and wear sterile vinyl or latex gloves.

S5. Describe in detail the surgical procedures.

Fish will be placed supine (ventral side up) in a plastic covered foam wedge to hold them securely. Commercially available "Stress Coat" will be applied to the wedge to minimize scale loss. The gills will be continuously perfused with an anesthetic solution (MS-222, 150 mg. L^{-1}, buffered to pH 7.0 with sodium bicarbonate) dripped through a perforated tube placed in the buccal cavity to maintain Stage 5 anesthesia. A small midventral incision will be made between the pectoral and pelvic fins with a sterile scalpel blade. The prototype tag will then pushed gently into the body cavity and the incision will be

sutured (surgical monofilament, three simple interrupted sutures). The area surrounding the sutures will be coated with surgical glue (VetBond) to minimize fluid exchange through the incision. These procedures follow common fishery agency protocols and have had other ACUPs approved for other projects.

S5a. Who will perform the surgery? List that person's name, training and/or experience (recent or other) with the surgical procedure.

The surgery will be performed Carl Schreck assisted by Kristin Berkenkamp.

S6. Describe sterile techniques that will be used during the survival procedures or provide a copy of a current standard operating procedure (SOP).

The person conducting the surgery will wear sterile vinyl or latex gloves. Scalpel blades will be used new (as sterile) for each surgery. Needles and suture material will be used as sterile from the manufacturer.

S7. Describe postoperative care procedures and practices (e.g., pain management, wound and infection control, diet augmentation, physical therapy, monitoring frequency, etc.) or provide a copy of a current SOP. Include the name of medications, doses, route, and frequency of administration.

Fish will be observed at least once each day during routine feeding, care and maintenance procedures at the FPGL. All fish at the FPGL are held in aerated, single-pass ground water, following standard operating procedures. Each tank will be cleaned of fecal material and excess food by siphon once daily. Fish will be observed for abnormal posture, swimming, gill ventilation, body coloration and condition of the sutures and surrounding tissue. Any fish showing abnormal behavior (e.g., inability to maintain posture, failure to respond to external stimuli, failure to show feeding behavior, labored or accelerated gill ventilation, abnormally light or dark body coloration or apparent inflammation of external tissue will be removed from the tank by dipnet and examined in detail. Any fish showing signs of ill health or sickness will be reported to the Attending Veterinarian (or designate).